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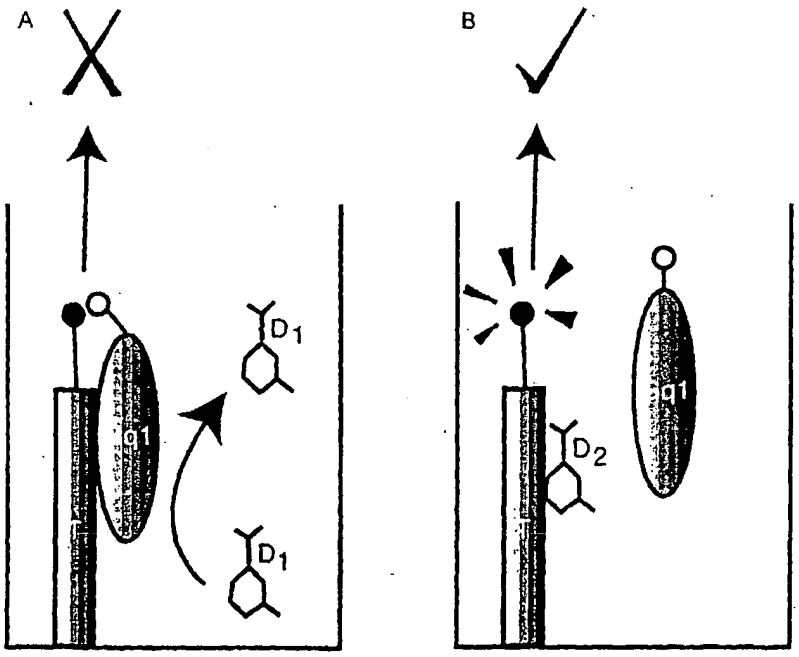
(54) Title: NUCLEIC ACID BINDING POLYPEPTIDES



(57) Abstract: We describe the use of a nucleic acid binding polypeptide capable of binding to one or more of telomeric, G-quadruplex, or G-quartet nucleic acid as an inhibitor of enzymatic activity, including a telomerase activity, a polymerase activity, an integrase activity and a gp120 activity. Telomerase assays as well as methods of identifying molecules capable of interacting with telomeric, G-quadruplex, or G-quartet nucleic acid are also described.

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Example 4. Inhibition of DNA Polymerase and Human Telomerase Activity by an Engineered Zinc Finger Protein that Binds G-Quadruplexes

The G-quadruplex nucleic acid structural motif is a target for designing molecules that could potentially modulate telomere length regulation, or have anti-cancer properties.

The engineered zinc finger protein (Gq1) binds with specificity to the intramolecular G-quadruplex formed by the human telomeric sequence 5'-(GGTTAG)5-3'. This Example demonstrates that Gq1 is able to arrest the action of a DNA polymerase, on a template containing a telomeric sequence. Inhibition occurs in a concentration-dependent manner, presumably by forming a G-quadruplex•protein complex. Furthermore, Gq1 inhibits the apparent activity of the enzyme telomerase *in vitro*, with an IC_{50} value of 74.3 ± 11.1 nM.

Using a DNA polymerase stop assay described previously (*18*) we study the effect that Gq1 binding has on the stability of the G-quadruplex structure. Furthermore, we use an *in vitro* assay to investigated if Gq1 can inhibit telomere synthesis by telomerase.

Materials and Methods

Preparation of Gq1

The glutathione S-transferase fusion of the zinc finger protein (Gq1) is purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4 Fast Flow (Pharmacia Biotech), as previously described.(*21*)

DNA Oligonucleotides

The following oligonucleotides are purchased from the Oswel DNA service (Southampton, UK): **Htemp**, 5'-(GTG CTT (GGG ATT)4ATG ATT ATG GAC GGC TGC GA)-3'; **13-mer**, 5'-(TCG CAG CCG TCC A; **TS**, AAT CCG TCG AGC AGA

GTT)-3'; **RP**, 5'-(GCG CGG (CTT ACC)$_3$CTA ACC)-3'; **ICT**, 5'-(AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT)-3'; **NT**, 5'-(ATC GCT TCT CGG CCT TTT)-3'; **TSR8**, 5'-(AAT CCG TCG AGC AGA GTT AG (GGT TAG)$_7$)-3'.

Annealing or Quadruplex Formation of Oligonucleotides

Oligonucleotides are diluted to 10μM in 50 mM Tris-HCl (pH 7.5) in the presence or absence of 100 mM KCl, as specified. Duplex annealing or quadruplex formation is carried out by heating samples to 95 °C, on a thermal heating block, and cooling to 4 °C at a rate of 2 °C/min.

Gel Mobility Shift Assay

Binding reactions are performed in a final volume of 10 μl, using 10 fmoles of labelled oligonucleotide and varying concentrations (0 to 1 μM) of purified Gq1 in binding buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 6 % glycerol, 100 μg/ml BSA, 1 μg/ml calf thymus DNA, 50 μM $ZnCl_2$ and 100 mM KCl). After incubating for 1 hr at room temperature, samples are loaded on a 8 % polyacrylamide (acrylamide:bisacrylamide = 33:1) non-denaturing gel. 0.5 X TB is used, both in the gel and as electrophoresis buffer. Electrophoresis is performed at 15 V/cm, for 2 h, at 4 °C. The gels are exposed in a phosphorimager cassette and imaged (Model 425E PhosphorImager; Molecular Dynamics, Inc). Bands are quantified using Imagequant software. The data are plotted as Ø (1-fraction of free DNA) versus protein concentration to determine the K_d, which is equal to the protein concentration at which half the free DNA is bound. Equilibrium dissociation constants (K_d) are extracted by non-linear regression using the program KaleidaGraph™ version 3.0.4 and the following equation:

$$Ø = [P] / \{K_d + [P]\}$$

where Ø denotes the fractional saturation of DNA (i.e. fraction of DNA complexed with the protein).(*14*)

Dimethyl Sulfate Protection Assay

DNA oligonucleotide **Htemp** is 5'-labelled with ^{32}P using T4 polynucleotide kinase (Sigma) and denatured by heating at 95 °C for 10 minutes. Annealing or quadruplex forming reactions are carried out as described above, in 50 mM Tris-HCl buffer (pH 7.5) in the presence or absence of 100 mM KCl. DMS protection is carried out as described by Maxam and Gilbert.(*25*) 1 µl of dimethylsulfate (DMS) is added to 0.2 pmoles of annealed DNA (either 'naked' or in complex with Gq1), in the presence of 1 µg/ml calf thymus DNA , at 4 °C, in 200 µl of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 6 % glycerol, 100 µg/ml BSA, 50 µM $ZnCl_2$ and KCl to 100 mM. The reaction is carried out for 5 min at room temperature and stopped by adding 1/4 volume of stop buffer containing 1 M -mercaptoethanol and 1.5 M sodium acetate, pH 7.0. The reaction products are ethanol precipitated twice and treated with 100 µl of 1 M piperidine at 90 °C for 30 min. The cleaved products are resolved on a 20 % PAGE polyacrylamide gel (8 M urea).

DNA Polymerase Stop Assay

This assay is adapted from the method described by Haiyong Han and co-workers.(*18*) The **13-mer** primer (10 µM) is 5'-labelled with ^{32}P and mixed with the template DNA **Htemp** (10 µM) and annealed as described above. The polymerase reaction is carried out in a final volume of 20 µl, using 20 fmoles of duplex (i.e. 1 nM) and various amounts of purified Gq1 in binding buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 6 % glycerol, 100 µg/ml BSA, 1 µg/ml calf thymus DNA, 50 µM $ZnCl_2$ and 100 mM KCl). Gq1 is incubated with the G-quadruplex of **Htemp** for 1 h at room temperature. The polymerase extension reaction is initiated by adding Klenow fragment of E. *coli* DNA polymerase I (exo^-) (46 nM) expressed and purified as previously described,(*8*) dATP, dTTP, dGTP, dCTP (1 mM each) and $MgCl_2$ (10 mM). Reactions are incubated at room temperature for 10 min, and then stopped by adding an equal volume of stop buffer (95 % formamide, 10 mM EDTA, 10 mM NaOH, 0.1 % xylene cyanol, 0.1 %

bromophenol blue). Extension products are separated on a 20 % PAGE / 8 M urea, and gels are visualised on a phosphorimager (Molecular Dynamics).

Measurement of Telomerase Activity

Telomerase activity is determined using the TRAPEZE detection kit (Intergen Company, U.S.A.), which is a PCR based assay originally described by Kim *et al.*(*17, 23*) The source of telomerase is S100 extracts from K562 cells (ATCC No. CCL-243) prepared as described previously.(*28*) The prepared cell extract is dialysed overnight at 4 °C using a 300 kDa Spectra/Por biotech cellulose ester (CE) dialysis membrane (Spectrum) to remove smaller proteins from the extract while retaining the 550 kDa telomerase complex. 2 µl of the above extract is used in each assay. Various concentrations of Gq1 are pre-incubated either with or without the cell extract (in triplicate), for 10 min at ambient temperature, prior to initiating the telomerase reaction. Telomerase/Gq1 reactions are initiated by the addition of dNTP's and the **TS** primer as per standard protocol. Control experiments are also carried out using GST protein which had been produced in the same way as Gq1 (data not shown). This control ensured that any telomerase inhibition observed is not due to any other molecule present in the purified protein sample. Reaction mixtures are incubated for 30 min at 30 °C, after which the samples are processed using a QIAquick Nucleotide Removal Kit (QIAGEN Ltd) which purifies DNA fragments by removing all the nucleotides and proteins (including Gq1) in the mixture. Pure DNA is eluted with PCR-grade water and samples for the PCR reactions are prepared by the addition of *Taq* polymerase, dNTP's, **TS** primer, **RP** primer, **NT** primer and the **ICT** template as per standard protocol. The samples are transferred to a GENEAMP 2400 thermocycler (Perkin Elmer) for PCR amplification of telomerase products (two-step cycle of 30 s at 94 °C, 30 s at 59 °C for 30 cycles). Samples are analysed using 8 % non-denaturing PAGE and quantitated using a Molecular Dynamics phosphorimager. The quantitation of telomerase products and the internal PCR control is as that described by Hamilton *et al.*(*17*) Data are normalised and plotted as telomerase activity against final Gq1 concentration. The IC_{50} value is estimated by fitting the data to the equation $y = 100 / (1 + (x / IC_{50})$.

Results and Discussion

To explore whether Gq1 is capable of inhibiting the copying of DNA by stabilising a telomeric G-quadruplex, a polymerase stop assay(*18, 37, 39*) is designed, as illustrated in Figure 9. The principle of the assay is to copy the template sequence **Htemp** that contains four consecutive human telomeric repeats 5'-(TTAGGG)-3'. The **13-mer** primer is annealed to the 3'-end of the template and can be extended by a DNA polymerase upon addition of the dNTPs. If complete extension of the primer occurs, a full length 50mer product is formed. However, factors that promote and stabilise intramolecular G-quadruplex formation may lead to a specific pause site on the template, resulting in the formation of a truncated 23mer product. The stop site corresponds to an adenine base on **Htemp** located 3' to the first guanine base involved in G-quadruplex formation. Before investigating the potential enzyme-inhibiting properties of Gq1, it is necessary to characterise the complex formed between the zinc fingers and an oligonucleotide that could serve as a template for a polymerase stop assay (**Htemp**; see Figure 9). The interaction is therefore studied by non-denaturing gel mobility shift analysis(*5, 6, 10, 32*) and by dimethyl sulphate (DMS) protection assays.(*36*)

Various concentrations of Gq1 are incubated with 5' ^{32}P-labelled-**Htemp** under conditions that promote and stabilise the G-quadruplex conformation (100 mM K^+). The resulting complexes are resolved on an 8% non-denaturing polyacrylamide gel. Figure 10A shows that, as Gq1 protein concentration is increased, there is a decrease in the free DNA (**Htemp**) and an increase in higher molecular weight protein-DNA complexes (**Htemp**·Gq1). The mobility shift data are fitted to a hyperbolic equation(*14*) to give an equilibrium dissociation constant (K_d) of 30 ± 10 nM (Figure 10B), which agrees with the Kd value of 34 nM previously obtained for the binding of Gq1 to a similar sequence.(*21*) No binding is observed for a control GST protein lacking the zinc finger fusion (data not shown).

The DNA template **(Htemp)** is expected to form a G-quadruplex secondary structure *in vitro* in the presence of 100 mM potassium ion concentration,(*18*)and a

dimethyl sulphate (DMS) protection assay is carried out to confirm this structure.(*36*) G-quadruplex formation requires Hoogsteen-type base pairing of guanines which protects N-7 of guanine against methylation, upon exposure to the potent methylating agent DMS. Quadruplexes therefore display characteristic patterns of protection against piperidine cleavage of the DNA backbone at methylated guanines.(*25*) Figure 11 shows that the critical, quadruplex-forming, guanines of the **Htemp** template are almost completely protected from cleavage at a K^+ concentration of 100 mM (Lane 3) as compared to a Tris buffer control (Lane 4). This is consistent with the Tris buffer lacking the metal cations required to stabilise quadruplexes. By contrast, the guanines that are not involved in quadruplex formation react strongly with DMS under both salt conditions. Similarly, when **Htemp** is incubated with 500 nM Gq1, in buffer containing 100 mM K^+, there is almost complete protection of the critical guanines. Since this set of conditions corresponds to a total band shift (lane 7, Figure 10A), which reflects complete complexation of the DNA by the protein, this suggests that Gq1 is binding specifically to the G-quadruplex formed within **Htemp**. These results are consistent with our previous observations reported for Gq1 binding to the human telomeric DNA sequence 5'-$(GGTTAG)_5$-3'.(*21*).

Having established that Gq1 binds to the G-quadruplex structure of **Htemp**, the polymerase stop assay is performed. The primer extension experiments are carried out with increasing concentrations of Gq1, using identical salt conditions to those in the mobility shift assay (i.e. 100 mM KCl; Figure 10A). A small amount of 23-mer pause-product is observed in the absence of Gq1, indicating the position of a G-quadruplex structure in the template (Figure 12A, lane 1). There is less 50mer product and more 23-mer with increasing Gq1 concentration with almost complete pausing at 1 μM Gq1 (Figure 12A, lane 5). The barrier to 50-mer DNA synthesis is quantitated as the ratio of the band intensities of paused extension product (23mer) to the total products in the lane.(*18*) This ratio is plotted against the Gq1 protein concentration in the primer extension reaction (Figure 12B). The termination of DNA synthesis at the pause site increases with Gq1 concentration until the effect saturates at ~500 nM Gq1. These results are consistent with Gq1 binding and stabilising the G-quadruplex to provide a block for

polymerase extension. Similar inhibition of DNA polymerase synthesis has also been shown for small organic molecules that bind G-quadruplex DNA(*18*).

Telomerase Activity Assays

To explore whether Gq1 has any influence on the *in vitro* activity of human telomerase, we employed the telomere repeat amplification protocol (TRAPEZE).(*23*) In the standard protocol, telomerase extends an oligonucleotide template (**TS** primer) to form discrete elongated telomeric products. These products are then amplified by PCR to facilitate their detection. Due to the limitations of the PCR reaction, whereby a minimum length of template is required for the reverse primer to hybridise and efficiently prime the PCR reaction, only products that have been elongated by four or more telomeric repeats are detected by this method. However, TRAPEZE allows a sensitive and linear response over the range of telomerase activity used in these studies,(*20*) and the inclusion of an internal amplification standard (IC) in each sample permits reproducible quantification. Although a PCR control carried out at 1 μM Gq1 shows that Gq1 does not directly inhibit *Taq* polymerase, controls have suggested that Gq1 does inhibit the PCR amplification of telomeric DNA (data in supplementary information). Therefore a modified TRAPeze assay has been employed, in which proteins are removed after the telomerase/Gq1 reactions, prior to PCR detection of telomeric repeats.

In the modified assay, telomerase/Gq1 extension reactions are first carried out with the exclusion of *Taq* polymerase and the PCR primers. Gq1 is subsequently removed by a protocol that ensures the removal of proteins, salts and unincorporated dNTP's from the reaction mixture. The purification exploits the denaturation of proteins with a high concentration of chaotropic salts, followed by adsorption of the telomeric DNA extension products onto a silica-gel membrane. After repeated washes to remove residual contaminants and salts, the adsorbed DNA is eluted in water and a PCR reaction carried out on the eluate to detect telomeric repeats. Using this modified protocol, telomerase activity is evaluated in the presence of Gq1 concentrations ranging from 0 to 375 nM (Figure 13, lanes 1-6). A control in which the telomerase extract is heat-inactivated at 90

°C for 10 min, confirmed that addition of telomeric repeats is due to enzyme activity in the extract (Figure 13, lane 7). In addition to the cell extract experiments, an eight-repeat telomeric oligonucleotide template (**TSR8**) is employed as a specific PCR control in the absence of telomerase (Figure 13; lanes 8 and 9). This control shows that even 2.5 μM Gq1 has a negligible effect on the PCR amplification of the 8 repeats of **TSR8**. The modified assay supports the conclusion that Gq1 is causing specific inhibition of telomerase-mediated extension of the **TS** primer. The telomerase inhibition by Gq1 is quantified as described previously,(*17*) and the IC_{50} value is calculated to be 77.1 ± 11.8 nM (Figure 14). This IC_{50} value is higher than the measured K_d of Gq1 for **Htemp** (30 ± 10 nM). This might be reflecting that a G-quadruplex structure formed during telomerase extension is less stable than the "free" G-quadruplex target used in the binding study.

Given the DNA polymerase stop-assay data, the molecular mechanism by which Gq1 inhibits extension by telomerase is likely to be through a direct interaction of Gq1 with a **TS** primer which has been extended by four or more telomeric repeats. This model is suported by the observation that Gq1 binds the G-quadruplex form of the sequence 5'-$(TTAGGG)_4$-3' in **Htemp** with a K_D = 30 ± 10 nM (Figures 10A, 11 and 12). Gq1 could therefore bind and stabilise telomeric G-quadruplex structures in the telomerase extension reaction resulting in the formation of a trapped Gq1·G-quadruplex·telomerase complex which disallows another molecule of **TS** primer from being extended by telomerase. Interestingly, in the telomerase assay carried out at the highest Gq1 concentration (375 nM; Figure 13, Lane 6), inhibition of telomerase extension seems to occur before four or more telomeric repeats have been added to the **TS** primer by telomerase. It is therefore possible that at higher levels of protein concentration, Gq1 may be binding to other telomeric secondary structures which may require less that four extended telomeric repeats to form.

Conclusion

Gq1 is an artificial protein that has been engineered to bind human telomeric G-quadruplex DNA. The primer extension studies presented here, using both telomerase and

Klenow fragment of E. *coli* DNA polymerase I, suggest that Gq1 can inhibit both the synthesis and copying of telomeric DNA sequences. Since this zinc finger protein has no detectable affinity for telomeric duplex DNA, Gq1 may prove an attractive probe for carrying out cell based studies, which will form the basis for future studies.

Example 5. The Effect of Gq1 on Trapeze Detection Assay

The experiments described here describe the effect of Gq1 on the PCR amplification step carried out in the standard TRAPEZE assay method.

To explore whether Gq1 had any influence on the *in vitro* activity of human telomerase we first employed the telomere repeat amplification protocol (TRAPEZE; *101*). In the standard protocol, telomerase extends an oligonucleotide primer (**TS** primer) to form elongated telomeric products. These products are then amplified by PCR to facilitate their detection. Due to the limitations of the PCR reaction whereby a minimum length of template is required for the reverse primer to hybridise to it and efficiently prime the PCR reaction, only products that have been elongated by four or more telomeric repeats are detected by this methodology. TRAPEZE allows a sensitive and linear response over the range of telomerase activity used in these studies (*102*), and the inclusion of an internal amplification standard (**IC**) in each sample permits reproducible quantification. The addition of the internal amplification standard also confirms that Gq1 does not interfere with *Taq* polymerase during amplification. A potential issue with this assay is that when examining molecules that interact directly with telomeric DNA, there exists the possibility of specific inhibition of the PCR amplification of telomeric DNA. Such an artefact would not be apparent from controls for the inhibition of Taq polymerase alone. We have examined the effect of Gq1 on the PCR amplification of **TSR8** which has a sequence identical to the **TS** primer extended with eight telomeric repeats (5'-AAT CCG TCG AGC AGA GTT AG(GGT TAG)8-3'). The results of this study are show in the gel in Figure 15 (lanes 12-18). As the concentration of Gq1 is increased from 0 to 200 nM there is a reduction in the intensity of fragments containing more than four telomeric repeats which correlates with the length of the telomere required to form an intramolecular

G-quadruplex structure which Gq1 can bind to. It is quite clear that Gq1 *does* inhibit the PCR amplification of **TSR8** in a concentration-dependent manner. That the amplification of the internal control (**IC**) is not affected by Gq1 is clear evidence that the inhibition is also sequence specific. Given this result, the apparent inhibiton of telomerase activity from TRAPeze assay could not be clearly interpreted and lead to the need for a modified assay where Gq1 is removed from the reaction mixture prior to the PCR amplification step (Figure 15 lanes 1-11). In Figure 15, Lane 1-3 show the extended telomerase product in the absence of Gq1 with the internal control marked as **IC**. As the concentration of Gq1 is increased up to 200 nM (Lanes 4-9) the longer telomeric extension products clearly appear to decrease in intensity. Lane 10 is a heat control to confirm that activity is due to telomerase, and lane 11 is a PCR control carried out at 1 mM Gq1 confirming that Gq1 does not inhibit with the *Taq* polymerase.

Preparation of Gq1

The glutathione S-transferase fusion of the zinc finger protein (Gq1) is purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4 Fast Flow (Pharmacia Biotech) ,as previously described.

DNA Oligonucleotides

The following oligonucleotides are purchased from the Oswel DNA service (Southampton, UK): **TS**, 5'-(AAT CCG TCG AGC AGA GTT)-3'; **RP**, 5'-(GCG CGG (CTT ACC)$_3$CTA ACC)-3'; **ICT**, 5'-(AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT)-3'; **NT**, 5'-(ATC GCT TCT CGG CCT TTT)-3'; **TSR8**, 5'-(AAT CCG TCG AGC AGA GTT AG (GGT TAG)$_7$)-3'.

Measurement of Telomerase Activity

Telomerase activity is determined using the TRAPEZE detection kit (Intergen), which is a PCR based assay originally described by Kim *et al.* (*3,32*) The source of

telomerase is S100 extracts from K562 cells (ATCC No. CCL-243) prepared as described previously (*104*). The prepared cell extract is dialysed overnight at 4 °C using a 300 kDa Spectra/Por biotech cellulose ester (CE) dialysis membrane (Spectrum) to remove smaller proteins from the extract while retaining the 550 kDa telomerase complex. 2 µl of the above extract is used in each assay. Gq1 at varying concentration is pre-incubated with or without the cell extract (in triplicate) for 10 min at ambient temperature prior to initiating the telomerase reaction by addition of dNTP's, **TS** primer 5'-(AAT CCG TCG AGC AGA GTT)-3', Taq polymerase, and PCR primers [PCR mix 1 containing **RP** + **ICT** + **NT** primers] as described in the TRAPEZE kit. Control experiments are also carried out at various concentration of Gq1 where instead of telomerase, a **TSR8** template containing 8 telomeric repeats is added. This control served to test if Gq1 specifically inhibits the PCR amplification of telomeric DNA.

All the above reaction mixtures are incubated for 30 min at 30 °C, after which the samples are transferred to a GENEAMP 2400 thermocycler (Perkin Elmer) for PCR amplification of telomerase products (two-step cycle of 30 s at 94 °C, 30 s at 59 °C for 30 cycles). Samples are analysed using 8 % non-denaturing PAGE and quantitated using a Molecular Dynamics phosphoimager.

Example 6. *In vivo* effects of Gq1 - transfection of mammalian cells with Gq1-GFP peptide

In order to ascertain the *in vivo* properties of Gq1, pilot experiments are carried out in which the genes for the three fingers of Gq1 are fused to the gene for Enhanced Green Fluorescent Protein (EGFP) (Figure 16). Plasmid contructs carrying these fusions are indroduced into mammalian cell lines by transient transfection, and any resulting phenotypic changes are monitored by fluorescence microscopy (Figures 17 to 20).

The sequence of the Gq1-NLS construct for GFP fusion is shown below. This construct contains the three zinc fingers from Gq1, an SV40 nuclear localisation signal

NCBI Nucleotide [Sign In] [Regis
PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Book
Search CoreNucleotide for Go Clear
Limits Preview/Index History Clipboard Details
Display GenBank Show 5 Send to Hide: sequence all but gene, CDS and mRNA
Range: from begin to end Reverse complemented strand Features: + Refresh

1: AX395585. Reports Sequence 28 from ...[gi:21066455] Links

Features Sequence

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DEFINITION  Sequence 28 from Patent WO0204488.
ACCESSION   AX395585
VERSION     AX395585.1  GI:21066455
KEYWORDS    .
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  ORGANISM  synthetic construct
            other sequences; artificial sequences.
REFERENCE   1
  AUTHORS   Choo,Y., Isalan,M., Liu,X., Patel,S. and Balasubramanian,S.D.
  TITLE     Nucleic acid binding polypeptides
  JOURNAL   Patent: WO 0204488-A 28 17-JAN-2002;
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            Services Limited (GB)
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       61 agggttag
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